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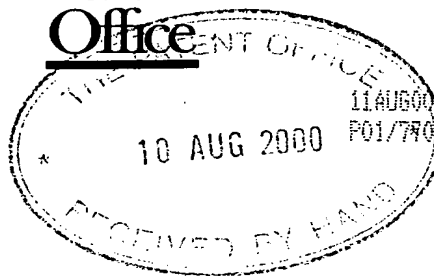
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0019744.2

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3.	Full name, address and postcode of the or of each applicant *(underline all surnames)*	Fermentas AB Graiciuno 8 LT-2028 Vilnius Lithuania
	Patents ADP number *(if you know it)*	
	If the applicant is a corporate body, give the country/state of its incorporation	7946809001

4.	Title of the invention	Nuclease

5.	Name of your agent *(if you have one)*	Page White & Farrer
	"Address for service" in the United Kingdom to which all correspondence should be sent *(including the postcode)*	54 Doughty Street London WC1N 2LS
	Patents ADP number *(if you know it)*	1255003

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11. I/We request the grant of a patent on the basis of this application.

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    Date **10 August 2000**

    PAGE WHITE & FARRER

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    Mr J N Daniels   020 7831 7929

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Nuclease

The present invention relates to a restriction endonuclease, a polynucleotide encoding such a restriction endonuclease, and processes for the preparation of such restriction endonucleases and polynucleotides.

Restriction endonucleases cleave DNA with extremely high sequence specificity and due to this property they have become indispensable tools in molecular biology and molecular medicine. Despite the fact that several hundred different restriction enzymes have been isolated from various bacterial strains, many specificities are still unavailable. This situation and the growing need for wider selection of available restriction enzymes with differing recognition sequences has stimulated efforts to produce artificial restriction enzymes.

The specificity of a restriction enzyme may be defined by several components: the DNA sequence recognised by the enzyme, its mode of cleavage, and its sensitivity to DNA modifications within the recognition sequence. Sequences recognised by an enzyme's star activity as well as an enzyme's preferences for certain nucleotides flanking the recognition sequence may also be attributed to specificity phenomena. Attempts have been made to alter the specificity of restriction endonucleases, especially to change an enzyme's recognition sequence specificity. Although certain progress in this field has been achieved (1), experiments that sought the alteration of enzyme specificity for its recognition sequence by mutagenesis have been unsuccessful.

After the tertiary structures for *Eco*RI and *Eco*RV enzymes became available and specific amino acid residues that form specific contacts to the bases of the recognition sequence were identified, site - specific mutagenesis experiments were undertaken aimed at producing enzyme variants with new specificities by altering these contacts. However, extensive mutagenesis studies covering all the amino acid residues that form specific contacts to the bases of the recognition sequence, carried out with *Eco*RI ((2-8) and *Eco*RV (9,10) revealed that none of the mutants had acquired a new specificity. Both by site - specific and saturating mutagenesis of residues involved in target recognition it was possible to produce some *Eco*RI and *Eco*RV mutants in which the coupling of recognition and catalysis is loosened, leading to a relaxed but not altered specificity of these mutants.

The same approach - rational protein design has been applied to engineering of restriction enzyme variants that attack modified substrates more readily than do the wild-type enzymes. This way, by eliminating one of the hydrophobic contacts between *Eco*RI and the methyl group of one thymine residue within its recognition sequence (GAA**T**TC) it was possible to create an *Eco*RI variant that could not discriminate between thymine and uracil at this position (7). Similarly, two *Eco*RV mutants have been constructed (11), one cleaving a uracil-containing substrate more rapidly than wild type enzyme, and a second showing preference for a recognition sequence in which the GATAT**p**C phosphate group is replaced by a methylphosphonate and hardly cleaving the unmodified substrate. A particular limitation of these newly created enzyme variants is that none of said substrate modifications occurs in native DNA, therefore these variants are unlikely to be useful as biochemical tools.

With a knowledge of tertiary enzyme structure protein engineering experiments were carried out to create enzymes that specifically recognise and cleave their recognition sequences when they are methylated. Site – specific mutagenesis of amino acid residues participating in DNA sequence recognition in *Bam*HI catalytic centre resulted in *Bam*HI variants which have lost their ability to cleave unmethylated GGATCC sequences by more than two orders of magnitude, while maintaining nearly wild-type levels of activity on the N6-methyladenine – containing sequence GGmATCC (12,13). Random mutagenesis combined with genetic screen for relaxed specificity mutants of *Fok*I restriction endonuclease resulted in isolation of *Fok*I mutants capable to cleave hemimethylated *Fok*I target sites in addition to those recognised by wild type enzyme (14).

Attempts based on site – directed mutagenesis have been undertaken to extend the specificity of restriction enzymes from six to eight, or even to ten base pairs by the creation of new base-specific contacts to base pairs that flank the recognition sequence. Several mutants of *Eco*RI and *Eco*RV have been created that differed in the cleavage rates of recognition sites with different flanking sequences much more than the wild type enzymes. After comparison of the cleavage rates of two *Eco*RV sites located in a different sequence context, which are cleaved at equal rates by the wild type enzyme, it was possible to identify *Eco*RV mutants that cleaved a site flanked by AT-rich sequences up to one order of magnitude more readily than a site flanked by GC-rich sequences (15, 16). Although *Eco*RV mutant variants showed more significant site preferences than the wild type enzyme, all attempts to alter restriction enzyme specificity from six nucleotides to eight were unsuccessful. Experimental data

demonstrate that even for the very well characterized restriction enzyme, such as *Eco*RV, the properties that determine specificity and selectivity are difficult to model on the basis of the available structural information, constituting this way the main limitation of all experiments based on rational protein design.

Another approach has been applied in experiments attempting to alter specificity of type IIS and type I restriction endonucleases. As these enzymes are composed of different domains or subunits, one of which is responsible for sequence specificity and another for catalysis, these enzymes are good targets for domain and subunit-swapping experiments in an attempt to transplant the specificity of one enzyme onto another one. Such experiments were quite successful. For example, by fusing the N-terminal half of the recognition subunit hsdS of the type I restriction enzyme *Sty*R124I recognising sequence GAAN$_6$RTCG with the C-terminal half of the hsdS subunit of *Eco*DXXI, which has the recognition sequence TCAN$_7$RTTC, a hybrid enzyme was obtained that had a new intermediate specificity GAAN$_6$RTTC (17). Transposon mutagenesis applied for *Eco*DXXI resulted in creation of novel mutant in which the specificity has been altered due to a Tn5 insertion into the middle of *hsdS* gene into an interrupted palindrome, TCAN$_8$TGA, in which the 5' half site of the wild type site is repeated in inverse orientation (18).

Series of experiments performed by fusing DNA binding domains of several proteins to the cleavage domain of *Fok*I restriction endonuclease resulted in creation of chimeric restriction endonucleases that cleave DNA at sites recognised by fused DNA binding domain (19 - 22).

All rational protein design experiments aiming to generate restriction enzymes with new specificities that were based on site-specific mutagenesis did not achieve the desired goal probably because the recognition of individual base pairs is determined by interactions with multiple amino acid residues and changes in specificity might require the simultaneous exchange of several amino acid residues at the protein-DNA interface.

The applicants have recognised that whilst a random mutagenesis approach has hitherto been applied (5, 12, 13, 14, 23, 24) to solve the problem of generating restriction enzymes with new specificities, this approach is quite limited in the absence of a useful selection method for the desired restriction enzyme.

The present invention now provides a process for producing a polynucleotide encoding a restriction endonuclease with an altered specificity, which process comprises:

    (a)   mutagenising a polynucleotide encoding a restriction endonuclease with specificity for a recognition sequence so as to produce one or more mutated polynucleotides; and

    (b)   isolating therefrom a polynucleotide encoding a mutated restriction endonuclease with specificity for an altered recognition sequence by selecting a polynucleotide which expresses a restriction endonuclease with methylase specificity for the altered recognition sequence.

The process of the present invention includes a powerful selection method for use in isolating restriction endonucleases with new specificities. This selection method exploits the ability of certain restriction endonucleases to exhibit methylation activity towards the recognition sequence in addition to the restriction endonuclease activities of sequence recognition and cleavage. In particular, selection of a polynucleotide which expresses a restriction endonuclease with altered recognition sequence methylase specificity can be carried out by cleaving the products of mutagenesis step (a), comprising mutated polynucleotides and wild-type polynucleotides, which have an unaltered recognition sequence methylase specificity. The polynucleotide is preferably DNA.

In a first embodiment the restriction endonuclease has methylase activity towards a target base in the recognition sequence and the altered recognition sequence comprises the recognition sequence altered in at least one nucleotide base.

In this embodiment there is therefore provided a process for producing a polynucleotide encoding a restriction endonuclease with an altered specificity, which process comprises:

    (a)   mutagenising a polynucleotide encoding a restriction endonuclease so as to produce a polynucleotide library comprising mutated polynucleotides, which restriction endonuclease has specificity for a recognition sequence and methylase activity towards a target base in the recognition sequence;

    (b)   incorporating each mutated polynucleotide into a polynucleotide vector to form a vector library, wherein the polynucleotide vector has a sub-sequence

comprising an altered sequence which comprises the recognition sequence altered in at least one nucleotide base, and a selection sequence overlapping the altered sequence to an extent which includes the target base;

(c) propagating the vector library to form a propagated library under conditions to permit restriction endonuclease catalysed polynucleotide methylase activity;

(d) treating the propagated library with a selection restriction endonuclease with specificity for the selection sequence and sensitivity to methylation in the selection sequence to cleave polynucleotides containing unmethylated selection sequences; and

(e) isolating therefrom an uncleaved polynucleotide encoding a restriction endonuclease with specificity for the altered sequence.

The restriction endonuclease to be mutagenised may be any suitable enzyme which additionally has methylase activity, preferably in the presence of S-adenosyl methionine so that the recognition sites in the vector, such as a plasmid vector carrying the gene for such a restriction endonuclease, are resistant at least to some extent to their own cleavage *in vitro* even without a gene for cognate DNA methylase being present in the host cell. Suitable endonucleases include those of type IV, such as *Eco*57I (25, 26) or *Gsu*I (27, 28). In enzymes of this type, mutations in the polynucleotide encoding the restriction endonuclease can affect both methylase and endonuclease specificity. Enzymes like this which cleave at their recognition sequence are good candidates for use in the process of the present invention. Other

candidates include type I or type III restriction enzymes; and
those type II restriction enzymes which contain in their amino
acid sequence one or more motifs characteristic of a DNA
methyltransferase. Such type II enzymes include *Bcg*I-type
enzymes (29), *Hae*IV-type enzymes (30), *Alo*I-type enzymes (31)
and *Cje*I type enzymes.

The polynucleotide encoding the restriction endonuclease may
be wild-type or may be itself a mutant variant including those
enzymes which possess endonuclease activity and have had their
methylation activity restored (see below), and enzymes which
favour cleavage over methylation such as *Eco*57I. In enzymes
of the latter class, it is possible to use cleavage-deficient
mutants as described in the specific description below as
targets for mutagenesis and subsequently reconstitute cleavage
activity to produce an altered restriction endonuclease in
accordance with the present invention.

The selection restriction endonuclease can also be any
suitable restriction endonuclease provided that it is
sensitive to methylation in the selection sequence and has a
selection sequence (*i.e.* its own recognition sequence) which
overlaps the altered sequence to an extent which includes the
target base. This is a preferred means by which selection of
the altered recognition sequence methylase specificity in the
mutant restriction endonuclease can be achieved. As set out
in the specific examples herein, the selection sequence may
overlap the altered sequence by a single base or by all bases,
as in the case of *Eco*57I and *Gsu*I. It is preferred that there
is at least a two base overlap, more preferably at least a
three base overlap. Overlap may also arise within a
recognition sequence, as is the case with *Alo*I which has the

recognition sequence GG**A**NNNNNNNGTTC (in which the **A** is the target base for methylase activity).

The precise method of mutagenesis is not critical to the invention and may be site-specific or random mutagenesis. The method of mutagenesis chosen will depend upon the need of any particular experimental protocol. Methods include use of mutagenic polymerase chain reaction (PCR), chemical mutagenesis, DNA shuffling and the use of a mutator host strain. The present invention is able to use non-specific or random mutagenesis methods because of the use of a powerful selection method to isolate mutant polynucleotides encoding desirable restriction endonuclease.

Once mutagenesis has taken place, each mutated polynucleotide may be incorporated into a polynucleotide vector by any conventional method. Any conventional vector such as a suitable cloning plasmid vector may be used provided that the vector has a sub-sequence comprising the altered sequence and a selection sequence overlapping the altered sequence as required herein. It is preferred that a plurality of such sub-sequences are present in the vector to achieve improved selection. The vector library may be propagated by any conventional method such as by transforming a suitable host such as a bacterial cell and growing the host in an appropriate growth medium. The bacterial cell may be a suitable strain of *E.coli*. The conditions under which the vector library is propogated must be such as to permit restriction endonuclease catalysed polynucleotide methylase activity. This methylase activity is required so as to permit methylation of the recognition sequences of the restriction endonucleases encoded by the vector library members. This

would normally be the case on propagation of a host cell such as *E.coli*.

In the step of treating the propagated library with a methylation sensitive selection restriction endonuclease, the aim is to cleave all polynucleotides containing unmethylated selection sequences so as to leave behind only those restriction endonucleases which successfully methylated the altered sequence as provided by the polynucleotide vector. It is preferred that this step is carried out on the propogated library after the library has been isolated from host cells. Whilst individual members could be treated in this way, it is advantageous for the propogated library to be pooled. Transformed host cells containing members of the propogated library therefore need not be grown individually but can themselves also be pooled or grown together.

The step of isolating from the treated propagated library an uncleaved polynucleotide is conveniently based on the significantly decreased transformation efficiency of cleaved polynucleotides to transform host cells such as bacteria. Other conventional methods are possible, including size-based separation of the uncleaved polynucleotides. During isolation of the uncleaved polynucleotide, it is convenient for this to be amplified either whilst present in the vector or following excision therefrom. Amplification of the uncleaved polynucleotide may be effected by any conventional means. Amplification is conveniently achieved by transformation of a host cell, propagation of that host cell and subsequent isolation therefrom of the uncleaved polynucleotide within the vector. The uncleaved polynucleotide would be expected to encode a mutant restriction endonuclease. Where a number of transformants are produced according to this method, the

vectors, such as plasmid DNA, from the transformants may be individually analysed by conventional methods to determine sequence information and restriction mapping behaviour of the mutant restriction endonucleases obtained thereby.

In a second embodiment of the present invention a method is provided whereby certain methylase deficient restriction enzymes can be used as target enzymes for mutagenesis by firstly restoring their methylase activity. In this embodiment, the restriction endonuclease is prepared by (a) selecting a methylase-deficient restriction endonuclease which has in its amino acid sequence a motif characteristic of a DNA methyltransferase; and (b) restoring methylase activity in the methylase deficient restriction endonuclease.

Preferably, the step of restoring the methylase activity comprises:

(a) mutagenising a polynucleotide encoding the methylase-deficient restriction endonuclease so as to produce a polynucleotide library comprising mutated polynucleotides, which restriction endonuclease has specificity for a recognition sequence;

(b) incorporating each mutated polynucleotide into a polynucleotide vector to form a vector library, which polynucleotide vector has the recognition sequence;

(c) propagating the vector library to form a propagated library under conditions to permit restriction endonuclease catalysed polynucleotide methylase activity;

(d) treating the propagated library with a restriction endonuclease with specificity for the recognition sequence to cleave polynucleotides containing an unmethylated recognition sequence; and

(e) isolating therefrom an uncleaved polynucleotide encoding a restriction endonuclease with methylase activity.

This process of restoring methylase activity operates in much the same way as the process for producing a polynucleotide encoding a restriction endonuclease with altered sequence specificity. Accordingly, the comments set out above particularly in relation to steps of mutagenising the polynucleotide and incorporating the mutated polynucleotide into a vector, propagating the vector library and finally isolating a desired restriction endonuclease therefrom apply equally here. The difference in the process arises from a different selection procedure for cleaving unwanted products of mutagenesis.

The method for cleaving unwanted mutagenesis products requires the activity of a restriction endonuclease with specificity for the recognition sequence to cleave those recognition sequences which are unmethylated. This is conveniently provided by the methylase deficient restriction endonuclease itself. By cleaving unmethylated recognition sequences, only those mutagenesis products encoding restriction endonucleases with restored methylase activity will be protected against cleavage.

Motifs characteristic of DNA methylases would be known to the skilled reader and typical examples are discussed in references 39 to 41.

The present invention will now be described in further detail, by way of example only, with reference to the accompanying drawings in which:

FIGURE 1 shows a schematic representation of a pUC19/eco57IR D78K plasmid;

FIGURE 2 shows a schematic representation of a pET21b/eco57IR D78K plasmid;

FIGURE 3 shows a schematic representation of a pET21b/eco57IR plasmid;

FIGURE 4 shows the results of agarose gel electrophoresis in determining substrate specificity of the *Eco*57I T862N mutant protein; and

FIGURE 5 shows the results of determining the *Eco*57I T862N cleavage site in a sequencing gel (A and B) and schematically (C).

Examples

Construction of *Eco*57I mutant variant with altered specificity

*Eco*57I restriction endonuclease is a bifunctional enzyme, which not only recognises and cleaves DNA sequence CTGA$\underline{A}$G(N)$_{16/14}$↓, but also methylates the second adenine within its recognition sequence (underlined) in the indicated DNA strand. Studies of *Eco*57I restriction endonuclease amino acid sequence revealed two sequence motifs 77PDX$_{13}$EXK and 811PDX$_{20}$DXK that are reminiscent of the catalytic/Mg^{2+} binding sites identified in some restriction endonucleases (0). Site directed mutagenesis experiments performed on both putative

catalytic centres of *Eco*57I restriction endonuclease have demonstrated that D78N, D78A, D78K mutations completely abolish the cleavage activity while retaining the methylation activity of restriction enzyme (0).

*Eco*57I rather cleaves than methylates its recognition sequence, therefore the gene for this restriction endonuclease is lethal for the host cell when introduced without cognate DNA methyltransferase. Since the mutant variants of this enzyme deficient in DNA cleavage and proficient in methylation of their recognition sequence were available at the time of conception of present invention these mutants were chosen as a model object to reduce the invention to practice. However, it is not always necessary to create cleavage deficient mutants to practice the disclosed invention. Due to unique structure of encoded proteins genes for the following Type II restriction endonucleases are non lethal to the host cells: *Bcg*I, *Hae*IV, *Alo*I (0), therefore selection for altered specificity of these enzymes according to disclosed method may be performed directly using the gene for the wild type enzyme.

*Gsu*I restriction endonuclease recognises DNA sequence CTGGAG(N)$_{16/14}\downarrow$ that differs in one nucleotide from that of *Eco*57I and is sensitive to the methylation of adenine in the indicated strand. Plasmids overproducing mutant version of *Eco*57I D78K are protected from cleavage with *Eco*57I, while they are sensitive to *Gsu*I cleavage. Thereby *Gsu*I restriction endonuclease is a good candidate to enrich and screen DNA libraries with *eco57IR* D78K mutagenized gene for the new specificity.

For a mutagenesis experiment, a gene encoding *Eco*57I D78K mutant protein was subcloned into pUC19 cloning vector, yielding this way plasmid pUC19/eco57IR D78K (Fig. 1). In parallel, plasmid pET21b/eco57IR D78K (Fig. 2) was constructed where the same gene was inserted into pET21b expression vector in the orientation coinciding with that of transcription from T7 promoter.

Plasmid pUC19/eco57IR D78K was used as a substrate for error-prone mutagenic PCR with standard M13/pUC direct (-46, 22-mer) and reverse (-46, 24-mer) sequencing primers (MBI Fermentas). 0.2 μM of each primer and 0.1 μg of plasmid DNA were taken into reaction and several mutagenic error-prone PCRs were performed as described in Leung et al. and Zhou et al. (0,0). 3.2 kb DNA fragments obtained in all mutagenic PCRs were combined together and agarose gel purified using DNA Extraction Kit (MBI Fermentas).

3.2 kb DNA fragments obtained in all mutagenic PCRs were subjected further to DNA shuffling as described in Stemmer at al. (0, 0). About 2 μg of gel purified 3.2 kb PCR fragment were incubated with 1 ng of DNAse I (Sigma) in the reaction buffer: 50mM Tris-HCl pH7.4, 100mM $MnCl_2$, at 30^0C for time points from 5 to 20 min. Degraded DNA fragments ranging from 50 to 500 bp were subsequently purified from agarose gel and taken into a self-assembly PCR reaction with no primers. All attempts to reassembly 3.2 kb PCR product were unsuccessful and resulted in a smeared DNA product that was somewhat smaller in size than 3.2 kb. Therefore this DNA was reamplified by nested PCR using M13/pUC sequencing primer and Primer#1, that annealed to the DNA sequence of *eco57IR* gene (see Fig. 1, positions 1495-1524).

Primer#1: 5'GCTTGATAGATAGTGGAGACAAAGTTAAAC3'

The obtained 1.6 kb PCR fragment was reamplified once more using Primer#1 and Primer#2 having introduced *Bam*HI site that annealed downstream the termination codon of *eco57IR* gene (positions 3065-3087 in Fig. 1).

Primer#2:   5'GTAAGTAGGGATCCAAAAGTCGG3'   (*Bam*HI   site   is underlined).

Following gel purification 1.6kb PCR fragment was digested with *Nde*I-*Bam*HI and a 1.5kb DNA fragment encompassing the C-terminal part of *eco57IR* gene was agarose gel purified. The fragment was ligated with *Nde*I-*Bam*HI digested and gel-purified pET21b/eco57IR D78K so as to substitute the wild type C-terminal portion of *eco57IR* gene with the mutagenized one. *E.coli* ER2566 cells were subsequently transformed with the ligation mixture by electroporation yielding this way a library of approximately 300,000 clones of *eco57IR* D78K gene mutants. All transformants after plating on ampicillin and IPTG (100μM) were incubated overnight at 37^{0}C and for another 24 hours at room temperature to ensure higher levels of expression of *Eco*57I mutant proteins, that should result in higher levels of methylation of plasmids encoding said proteins. Afterwards all transformants were pooled, total plasmid DNA was isolated and subjected to digestion with *Gsu*I. Plasmid pET21b/eco57IR D78K has six recognition sites for *Gsu*I, five of them originating from the vector molecule (see Fig. 2). Therefore all plasmids where *Gsu*I recognition sequences are non-methylated were fragmented. The only plasmids that could survive the *Gsu*I digestion should be the

ones where *Gsu*I recognition sequences become methylated due to the altered specificity of *Eco*57I D78K protein. *E.coli* ER2566 cells were subsequently transformed with a digestion mixture by standard $CaCl_2$ transformation techniques and plated on ampicillin, IPTG (100μM) plates. Individual plasmid DNAs were isolated from about 100 transformants obtained thereby and subjected to restriction endonuclease mapping. A restriction map of eleven plasmids coincided with that of pET21b/eco57IR D78K and all of them when isolated from IPTG induced cells were to a certain extent protected from both *Gsu*I and *Eco*57I cleavage. These data suggest that these plasmids encode *Eco*57I D78K mutant variant with the new specificity 5'CTGPuAG3'.

Seven plasmids were sequenced in order to deduce what type of mutations result in the alteration of *Eco*57I specificity. According to sequencing data all mutant proteins could be grouped into four types:

1. T862N (one clone)
2. Q777R, T862S (one clone)
3. E673, N720, T862S (four clones)
4. E673G, N720D, K770N, T862S (one clone)

Sequencing results indicate that all mutant variants had the substitution of threonine at the position 862, that should be the only substitution in all cases responsible for altered specificity. In order to test that selected mutant variants of *Eco*57I exhibit not only altered specificity of methylation, but altered cleavage activity as well, the latter has been restored in the mutant variant with single mutation T862N. For this purpose 1509 bp DNA fragment excised with *Mlu*I-*Bsp*1407I from plasmid pET21b/eco57IR D78K T862N was substituted by the

same fragment from the plasmid pET21b/eco57IR encoding the wild type *eco57IR* gene (Fig.3). *E.coli* ER2566 cells containing both *gsuIM* and *eco57IM* genes provided on compatible plasmids were transformed with ligation mixture and transformants were analysed by restriction mapping with *TatI* having an additional recognition site overlapping the D78K mutation. Restriction endonuclease activity was detectable in the crude cell extracts prepared from IPTG induced cells carrying plasmid where the cleavage domain of *Eco57I* T862N mutant variant has been restored. *Eco57I* T862N mutant and double mutant *Eco57I* D78K T862N proteins were isolated and purified in order to determine more precisely their biochemical properties.

Purification of Eco57I mutant proteins

Recombinant plasmids that were used for overexpression of *Eco57I* T862N and *Eco57I* D78K T862N mutant proteins, pET21b/eco57IR T862N and pET21b/eco57IR D78K T862N (see Fig. 2; restriction maps of both plasmids are identical except an additional *TatI* recognition site in pET21b/eco57IR D78K T862N that overlaps D78K), employed an inducible T7 promoter expression system.

Induction scheme for said proteins was as follows: *E.coli* ER2566 cells were transformed with relevant overexpressing plasmids. In the case of pET21b/eco57IR T862N host cell DNA was pre-methylated by introducing both *gsuIM* and *eco57IM* genes on compatible plasmids prior to transformation. Transformants were cultivated overnight in liquid LB medium supplemented with ampicillin (50mg/l) at 37^0C. The overnight culture was added to fresh LB medium supplemented with ampicillin (50mg/l) and propagated at 37^0C till mid log phase (OD_{600} =0.7). IPTG was

then added to the 500 µM final concentration and cultivation was carried out at 30°C for another 4 hours. The cells then were harvested by centrifugation and proteins were purified as follows:

1. Purification of Eco57I T862N mutant protein: All the following procedures were performed either on ice or at 4°C. Cell biomass was resuspended in Buffer A (10mM K_2HPO_3 pH7.0, 1mM EDTA, 7mM 2-mercaptoethanol) supplemented with 300mM NaCl at the ratio 4 ml buffer/1g cell biomass and broken by sonication (22kHz, 100W) for 5-8 min./100 ml suspension. Nucleic acids were eliminated by addition to sonicated suspension of poliethylenimine to 1% final concentration and by centrifugation at 10,000 rpm (Beckman JA10 rotor) for 10 min. Supernatant was collected and dry ammonium sulphate was added while slow mixing to 60% saturation. Proteins were precipitated by centrifugation at 10,000 rpm (Beckman JA10 rotor) for 10 min and collected pellet was dissolved in Buffer A supplemented with 200mM NaCl at the ratio 1-2 ml/1g pellet. Protein suspension was then dialyzed against 30-50 times higher volume of Buffer A supplemented with 200mM NaCl. Resulting protein extract was then loaded onto a phosphocellulose P11 (Whatman) column equilibrated with Buffer A supplemented with 200mM NaCl at the ratio 2-4 ml sorbent/1g cell biomass. The column was washed with two column volumes of Buffer A supplemented with 200mM NaCl and a linear gradient of ten column volumes from 0.2M to 1M of NaCl dissolved in Buffer A was applied with the flow speed 10 ml/cm² per hour. The enzyme eluted at 0.29-0.4M NaCl and was pooled. Pooled fractions were then dialyzed against 20-30 times higher volume of Buffer A overnight and loaded onto a Q Sepharose (Amersham

Pharmacia Biotech) column equilibrated with Buffer A at the ratio 0.5-0.9 ml sorbent/1g cell biomass. The column was washed with two column volumes of Buffer A and a linear gradient of ten column volumes from 0.0 M to 0.3M of NaCl dissolved in Buffer A was applied with the flow speed 10 ml/cm^2 per hour. The enzyme eluted at 0.165-0.19M NaCl and was pooled. Pooled fractions were then dialyzed against 20-50 times higher volume of Buffer A supplemented with 100mM NaCl overnight and loaded onto a Blue Sepharose (Amersham Pharmacia Biotech) column equilibrated with Buffer A supplemented with 100mM NaCl at the ratio 0.3-0.8 ml sorbent/1g cell biomass. The column was washed with two column volumes of Buffer A supplemented with 100mM NaCl and a linear gradient of ten column volumes from 0.1M to 0.7M of NaCl dissolved in Buffer A was applied with the flow speed 10 ml/cm^2 per hour. The enzyme eluted at 0.44-0.57M NaCl and was pooled. Pooled fractions were then dialyzed against 20-50 times higher volume of Buffer A overnight and loaded onto a DEAE-52 Cellulose (Whatman) column equilibrated with Buffer A at the ratio 0.3-0.8 ml sorbent/1g cell biomass. The column was washed with two column volumes of Buffer A and a linear gradient of ten column volumes from 0.0M to 0.3M of NaCl dissolved in Buffer A was applied with the flow speed 10 ml/cm^2 per hour. The enzyme eluted at 0.06-0.1M NaCl, was pooled and immediately loaded onto a hydroxyl apatite (Calbiochem) column equilibrated with Buffer A supplemented with 100mM NaCl at the ratio 0.3-0.8 ml sorbent/1g cell biomass. The column was washed with two column volumes of Buffer A supplemented with 100mM NaCl and a linear gradient of ten column volumes from 0.01M to 0.2M of NaCl dissolved in Buffer A was

applied with the flow speed 10 ml/cm^2 per hour. The enzyme eluted at 0.11-0.16M NaCl and was pooled. Pooled fractions were then dialyzed against 10-20 times higher volume of Storage Buffer (10mM K_2HPO_4 pH7.4, 100mM NaCl, 1mM EDTA, 7mM 2-mercaptoethanol, 50% v/v glycerol). The above purification scheme yielded apparently homogenous protein preparation with the molecular weight of about 108 kDa as confirmed by Coomasie blue R-250 stained SDS-PAGE gel electrophoresis. Protein concentration was measured according to Bradford and was found to be 0.5 mg/ml with specific activity 2u/µg of protein when expressed in restriction endonuclease activity units (determined as described in MBI Fermentas Catalogue).

2. Purification of *Eco*57I D78K T862N mutant protein: All the following procedures were performed either on ice or at 4^{0}C. Cell biomass was resuspended in Buffer A (10mM K_2HPO_3 pH7.0, 1mM EDTA, 7mM 2-mercaptoethanol) supplemented with 200mM NaCl at the ratio 4 ml buffer/1g cell biomass and broken by sonication (22kHz, 100W) for 5-8 min./100 ml suspension. Nucleic acids were eliminated by addition to sonicated suspension of polyethylenimine to 1% final concentration and by centrifugation at 10,000 rpm (Beckman JA10 rotor) for 10 min. Supernatant was collected and dry ammonium sulphate was added while slowly mixing to 60% saturation. Proteins were precipitated by centrifugation at 10,000 rpm (Beckman JA10 rotor) for 10 min and collected pellet was dissolved in Buffer A supplemented with 200mM NaCl at the ratio 1-2 ml/1g pellet. Protein suspension was then dialyzed against 30-50 times higher volume of Buffer A supplemented with 200mM NaCl. Resulting protein extract was then loaded onto a phosphocellulose P11 (Whatman)

column equilibrated with Buffer A supplemented with 200mM NaCl at the ratio 2-4 ml sorbent/1g cell biomass. The column was washed with two column volumes of Buffer A supplemented with 200mM NaCl and a linear gradient of ten column volumes from 0.2M to 1M of NaCl dissolved in Buffer A was applied with the flow speed 10 ml/cm^2 per hour. The enzyme eluted at 0.29-0.37M NaCl and was pooled. Pooled fractions were then dialyzed against 20-30 times higher volume of Buffer A overnight and loaded onto a Q Sepharose (Amersham Pharmacia Biotech) column equilibrated with Buffer A at the ratio 0.5-0.9 ml sorbent/1g cell biomass. The column was washed with two column volumes of Buffer A and a linear gradient of ten column volumes from 0.0 M to 0.3M of NaCl dissolved in Buffer A was applied with the flow speed 10 ml/cm^2 per hour. The enzyme eluted at 0.16-0.19M NaCl and was pooled. Pooled fractions were then dialyzed against 20-50 times higher volume of Buffer A supplemented with 100mM NaCl overnight and loaded onto a Blue Sepharose (Amersham Pharmacia Biotech) column equilibrated with Buffer A supplemented with 100mM NaCl at the ratio 0.3-0.8 ml sorbent/1g cell biomass. The column was washed with two column volumes of Buffer A supplemented with 100mM NaCl and a linear gradient of ten column volumes from 0.1M to 0.7M of NaCl dissolved in Buffer A was applied with the flow speed 10 ml/cm^2 per hour. The enzyme eluted at 0.48-0.65M NaCl and was pooled. Pooled fractions were then dialyzed against 20-50 times higher volume of Buffer A overnight and loaded onto a DEAE-52 Cellulose (Whatman) column equilibrated with Buffer A at the ratio 0.3-0.8 ml sorbent/1g cell biomass. The column was washed with two column volumes of Buffer A and a linear gradient of ten

column volumes from 0.0M to 0.3M of NaCl dissolved in Buffer A was applied with the flow speed 10 ml/cm^2 per hour. The enzyme eluted at 0.06-0.09M NaCl, was pooled and immediately loaded onto a Bordo Sepharose (MBI Fermentas) column equilibrated with Buffer A supplemented with 100mM NaCl at the ratio 0.3-0.8 ml sorbent/1g cell biomass. The column was washed with two column volumes of Buffer A supplemented with 100mM NaCl and a linear gradient of ten column volumes from 0.1M to 0.8M of NaCl dissolved in Buffer A was applied with the flow speed 10 ml/cm^2 per hour. The enzyme eluted at 0.32-0.43M NaCl and was pooled. Pooled fractions were then dialyzed against 10-20 times higher volume of Storage Buffer (10mM K$_2$HPO$_4$ pH7.4, 100mM NaCl, 1mM EDTA, 7mM β-mercaptoethanol, 50% v/v glycerol). The above purification scheme yielded apparently homogenous protein preparation with the molecular weight of about 108 kDa as confirmed by Coomasie blue R-250 stained SDS-PAGE gel electrophoresis. Protein concentration was measured according to Bradford and was found to be 0.7 mg/ml with specific activity 3.5u/μg of protein when expressed in DNA methyltransferase units (determined as described in MBI Fermentas Catalogue).

Recognition specificity, cleavage mode and methylation specificity were determined for purified Eco57I mutant proteins.

Determination of the recognition specificity and cleavage site of Eco57I T862N mutant protein

To determine the substrate specificity of the Eco57I T862N mutant variant it was incubated with 1 μg λ phage DNA for one hour at 37°C in 50 μl of MBI Fermentas B+ buffer (10mM Tris-HCl, pH 8.5, 100mM KCl, 10mM MgCl$_2$ and 0,1 mg/ml BSA) containing 0.01mM SAM (Fig. 4). In parallel the same substrate was incubated with the following enzymes and enzyme mixtures: Eco57I (recognition sequence CTGAAG), GsuI (recognition sequence CTGGAG), Eco57I+GsuI, Eco57I+GsuI+Eco57I T862N. Electrophoretic analysis of restriction digests confirmed that the mutant variant cleaved DNA at both Eco57I and GsuI recognition sites, thereby its recognition sequence being 5' CTGPuAG3'.

pACYC 177 DNA was used as a template to characterize the cleavage position of Eco57I T862N mutant protein, namely the sites located at the positions 454 (GsuI) and 3798 (Eco57I). Four dideoxy sequencing reactions (G, A, T, C, respectively) using [α-33P]dATP were carried out. The same template and primers were used in the fifth non-terminating reaction, which also included T7 DNA polymerase, dNTPs and [α-33P]dATP. The extension reaction was heat inactivated, radiolabelled DNA was incubated with Eco57I T862N and, subsequently, the reaction mix was divided into two. One sample was treated with T4 DNA polymerase. Both samples were diluted with sequencing dye and loaded on a standard sequencing gel together with the dideoxy sequencing reactions. The cleavage site of Eco57I T862N was determined by comparison of dideoxy sequencing ladders with

both the fragments generated by *Eco*57I T862N cleavage and DNA fragments obtained after the T4 DNA polymerase action on the digestion product.

Results of the determination *Eco*57I T862N cleavage site presented in Fig. 5 indicate that *Eco*57I T862N mutant protein cleaves DNA 16 and 14 nucleotides away from the site CTGGAG (*Gsu*I) and 16 or 17 and 14 or 15 nucleotides away from the site CTGAAG (*Eco*57I), thereby exhibiting degenerate cleavage point for one of the sequences recognised.

The key to Figure 5 is as follows:

Panel A: Reactions through *Gsu*I recognition site.

Panel B: Reactions through *Eco*57I recognition site.

G, A, T, C – sequencing ladders

R – Extension products digested with *Eco*57I T862N

T4 – Extension products digested with *Eco*57I T862N after reaction with T4 DNA polymerase

Panel C: Schematic representation of cleavage point determination of *Eco*57I T862N mutant protein:

Determination of the methylation specificity of *Eco*57I T862N and *Eco*57I D78K T862N mutant proteins

The modification specificity was determined by using two oligodeoxyribonucleotide (30-mer and 36-mer) duplex DNA fragments containing the substrate sequence for the *Gsu*I (#1) and *Eco*57I (#2) restriction endonucleases (presented in bold):

#1:

      5'-AGTT**CTGGAG**CATCGTTCACCGGTTACAAC
      CTCTTCAA**GACCTC**GTAGGCAATGGCCAATGTTGGT-5'
#2:

      5'-AGTT**CTGAAG**CATCGTTCACCGGTTACAAC
      CTCTTCAA**GACTTC**GTAGGCAATGGCCAATGTTGGT-5'

The methylation reaction was performed in 50µl of B+ reaction
buffer (MBI Fermentas) containing 100 pmol of the synthetic
duplexes and 30µM of [^{3}H-methyl]-AdoMet (67 Ci/mmol). 10
activity units as defined above of the *Eco*57I mutant proteins
were added to relevant reaction mixtures and incubated for 3h
at 37^{0}C. Reactions were stopped by adding 20µl of sequencing
dye, denatured by heating for 5 min. and loaded on 15%
polyacrylamide gel under denaturing conditions.

To determine the capacity of *Eco*57I mutant proteins to modify
different strands of the substrate, top and bottom strands of
oligonucleotide duplex after PAGE were identified by ethidium
bromide staining, excised from the gel and incorporated ^{3}H-
radioactivity was estimated by liquid scintillation counting.
Results are presented in the table below:

No	Oligonucleotide	H3-radioactivity	Enzyme
	*Eco*57I sequences		
1	5'-CTGAAG-3'	21846	*Eco*57I (MBI Fermentas)
2	3'-GACTTC-5'	406	
3	5'-CTGAAG-3'	17886	*Eco*57I T862N
4	3'-GACTTC-5'	198	
5	5'-CTGAAG-3'	10170	*Eco*57I D78K T862N
6	3'-GACTTC-5'	278	
	*Gsu*I sequences		
7	5'-CTGGAG-3'	13206	*Gsu*I (MBI Fermentas)
8	3'-GACCTC-5'	664	
9	5'-CTGGAG-3'	30574	*Eco*57I T862N
10	3'-GACCTC-5'	980	
11	5'-CTGGAG-3'	32151	*Eco*57I D78K T862N
12	3'-GACCTC-5'	920	

Obtained data indicate, that mutant variants of *Eco*57I protein have altered methylation specificity: 5' CTGPuAG3', and, like *Eco*57I and *Gsu*I restriction endonucleases, they methylate only one DNA strand.

*Gsu*I is not the only one example of restriction enzymes that may be used for selection of *Eco*57I mutant variants according to disclosed method. For example, *Bgl*II, recognising DNA sequence AGATCT is sensitive to methylation produced by *Eco*57I when their sequences overlap: CTGA<u>AGATCT</u> (*Eco*57I recognition sequence is underlined, modified base is bolded), therefore it may be used to select for the set of altered specificities of R.*Eco*57I listed below, provided that such sequences differing in at least one nucleotide from the one recognised by *Eco*57I and overlapping the recognition sequence for *Bgl*II are present

in the cloning vehicle used for *Bgl*II digestion selection step (all sequences that would be recognised by newly created specificity of *Eco*57I mutant variants are underlined, methylated adenine is presented in bold and altered bases are in lower case):

aTG**AA**GATCT
tTGA**A**GATCT
gTGA**A**GATCT
CaG**AA**GATCT
CcG**AA**GATCT
CgG**AA**GATCT
CTa**AA**GATCT
CTc**AA**GATCT
CTt**AA**GATCT
CTGt**A**GATCT
CTGg**A**GATCT
CTGc**A**GATCT

Likewise, *Hind*III restriction endonuclease, recognising sequence AAGCTT is sensitive to the methylation, produced by *Alo*I restriction endonuclease (recognition sequence GGA**N**NNNNNGTTC, methylated nucleotide in the top strand is presented in bold), when their recognition sequences overlap: GG**A**AGCTTNGTTC, therefore it may be used to select for the following set of altered specificities of *Alo*I, provided that such sequences differing in at least one nucleotide from the one recognised by *Alo*I and overlapping the recognition sequence for *Hind*III are present in the cloning vehicle used for *Hind*III digestion selection step (all sequences that would be recognised by newly created specificity of *Alo*I mutant variants are underlined, methylated adenine is presented in bold):

aGAAGCTTNGTTC

tGAAGCTTNGTTC

cGAAGCTTNGTTC

GaAAGCTTNGTTC

GtAAGCTTNGTTC

GcAAGCTTNGTTC

GGAAGCTTNaTTC

GGAAGCTTNtTTC

GGAAGCTTNcTTC

GGAAGCTTNGaTC

GGAAGCTTNGgTC

GGAAGCTTNGcTC

GGAAGCTTNGTaC

GGAAGCTTNGTgC

GGAAGCTTNGTcC

GGAAGCTTNGTTa

GGAAGCTTNGTTg

GGAAGCTTNGTTt

The present method allows selection for mutant variants recognizing new sequences that differ not only in single nucleotide from that of the wild type enzyme but also for mutants that would recognize degenerate sequences or sequences differing in several nucleotides, especially if mutants of altered specificity are taken into the next round of mutagenesis and digestion-selection.

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CLAIMS:

1.   A   process   for   producing   a   polynucleotide   encoding   a
restriction endonuclease with an altered specificity, which
process comprises:

    (a) mutagenising   a   polynucleotide   encoding   a
         restriction endonuclease with specificity for a
         recognition sequence so as to produce one or more
         mutated polynucleotides; and

    (b) isolating   therefrom   a   polynucleotide   encoding   a
         mutated restriction endonuclease with specificity
         for an altered recognition sequence by selecting a
         polynucleotide   which   expresses   a   restriction
         endonuclease with methylase specificity for the
         altered recognition sequence.

2.   A process according to claim 1, wherein the restriction
endonuclease has methylase activity towards a target base in
the recognition sequence, and the altered recognition sequence
comprises the recognition sequence altered in at least one
nucleotide base.

3.   A process according to claim 2, wherein the restriction
endonuclease comprises a type I restriction endonuclease, a
type II restriction endonuclease containing in its amino acid
sequence a motif characteristic of a DNA methyltransferase, a
type III restriction endonuclease or a type IV restriction
endonuclease.

4.   A process according to claim 3, wherein the restriction
endonuclease comprises Eco57I.

5.   A process according to claim 3, wherein the restriction endonuclease comprises BcgI, HaeIV or AloI.

6.   A process according to any one of claims 2 to 5, wherein the step of isolating the polynucleotide encoding the mutated restriction endonuclease comprises:

    (i)   incorporating each mutated polynucleotide from step (a) into a polynucleotide vector to form a vector library, wherein the polynucleotide vector has a sub-sequence comprising the altered sequence, and a selection sequence overlapping the altered sequence to an extent which includes the target base;

    ii)   propagating the vector library to form a propagated library under conditions to permit restriction endonuclease catalysed polynucleotide methylase activity;

    iii)treating the propagated library with a selection restriction endonuclease with specificity for the selection sequence and sensitivity to methylation in the selection sequence to cleave polynucleotides containing unmethylated selection sequences; and

    isolating therefrom an uncleaved polynucleotide encoding the mutant restriction endonuclease.

7.   A process according to claim 6, wherein the selection restriction endonuclease comprises GsuI or BglII when the restriction endonuclease comprises Eco57I.

8.   A process according to claim 6, wherein the selection restriction endonuclease comprises HindIII when the restriction endonuclease comprises AloI.

9.   A process according to any one of claims 2 to 8, wherein the step of propagating the vector library comprises transforming a host cell therewith and propagating the host cells.

10.   A process according to claim 9, which further comprises isolating the propagated library from the host cells following their propagation.

11.   A process according to any one of the preceding claims, wherein the restriction endonuclease is prepared by (a) selecting a methylase-deficient restriction endonuclease which has in its amino acid sequence a motif characteristic of a DNA methyltransferase; and (b) restoring methylase activity in the methylase deficient restriction endonuclease.

12.   A process according to claim 11, wherein the step of restoring methylase activity comprises:

    (a) mutagenising a polynucleotide encoding the methylase-deficient restriction endonuclease so as to produce a polynucleotide library comprising mutated polynucleotides, which restriction endonuclease has specificity for a recognition sequence;

    (b) incorporating each mutated polynucleotide into a polynucleotide vector to form a vector library, which polynucleotide vector has the recognition sequence;

    (c) propagating the vector library to form a propagated library under conditions to permit restriction endonuclease catalysed polynucleotide methylase activity;

(d) treating the propagated library with a restriction endonuclease with specificity for the recognition sequence to cleave polynucleotides containing an unmethylated recognition sequence; and

(e) isolating therefrom an uncleaved polynucleotide encoding a restriction endonuclease with methylase activity.

13. A process for producing a restriction endonuclease with an altered specificity, which comprises producing a polynucleotide encoding a restriction endonuclease in accordance with any one of the preceding claims, expressing the restriction endonuclease and harvesting the restriction endonuclease.

14. A polynucleotide encoding a restriction endonuclease with altered specificity, obtainable from a process according to any one of claims 1 to 12.

15. A polynucleotide which encodes a restriction endonuclease which comprises an Eco57I or mutant or variant thereof with specificity for a recognition sequence comprising 5'-CTGPuAG-3'.

16. A restriction endonuclease obtainable from a process according to claim 13.

17. A restriction endonculease which comprises an Eco57I or mutant or variant thereof with specificity for a recognition sequence comprising 5'-CTGPuAG-3'.

Abstract

Nuclease

A process for producing a polynucleotide encoding a restriction endonuclease with an altered specificity, which process comprises:

    (a)   mutagenising a polynucleotide encoding a restriction endonuclease with specificity for a recognition sequence so as to produce one or more mutated polynucleotides; and

    (b)   isolating therefrom a polynucleotide encoding a mutated restriction endonuclease with specificity for an altered recognition sequence by selecting a polynucleotide which expresses a restriction endonuclease with methylase specificity for the altered recognition sequence.
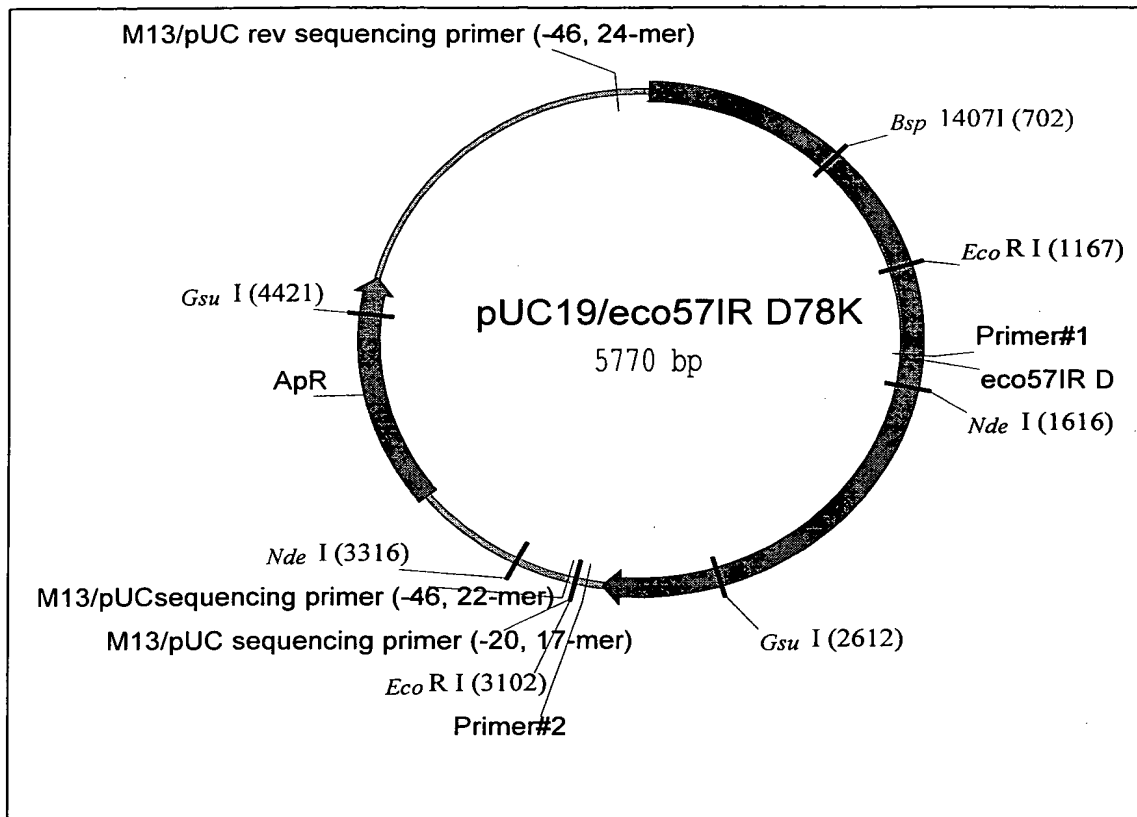
Fig. 1. Schematic representation of pUC19/eco57IR D78K plasmid. Binding sites of all primers used in PCR experiments are indicated in the picture.
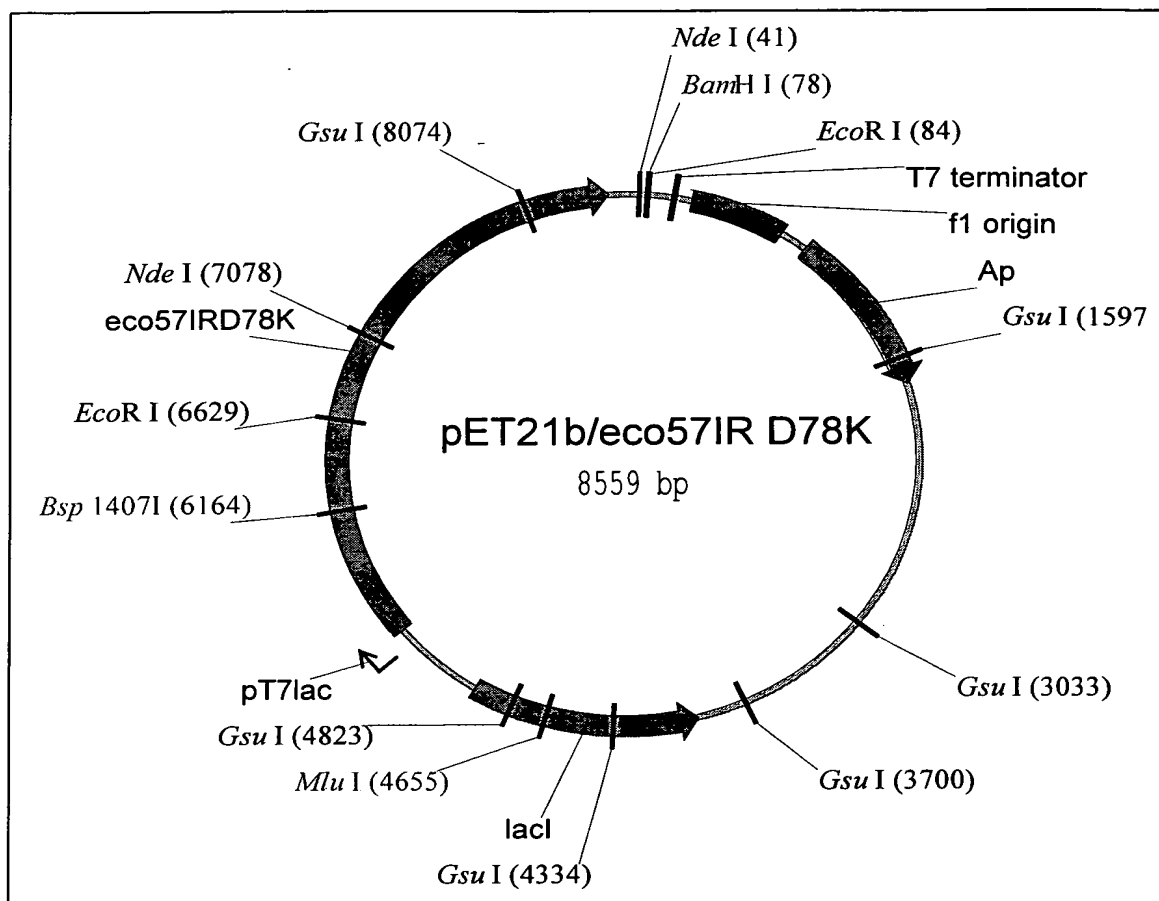
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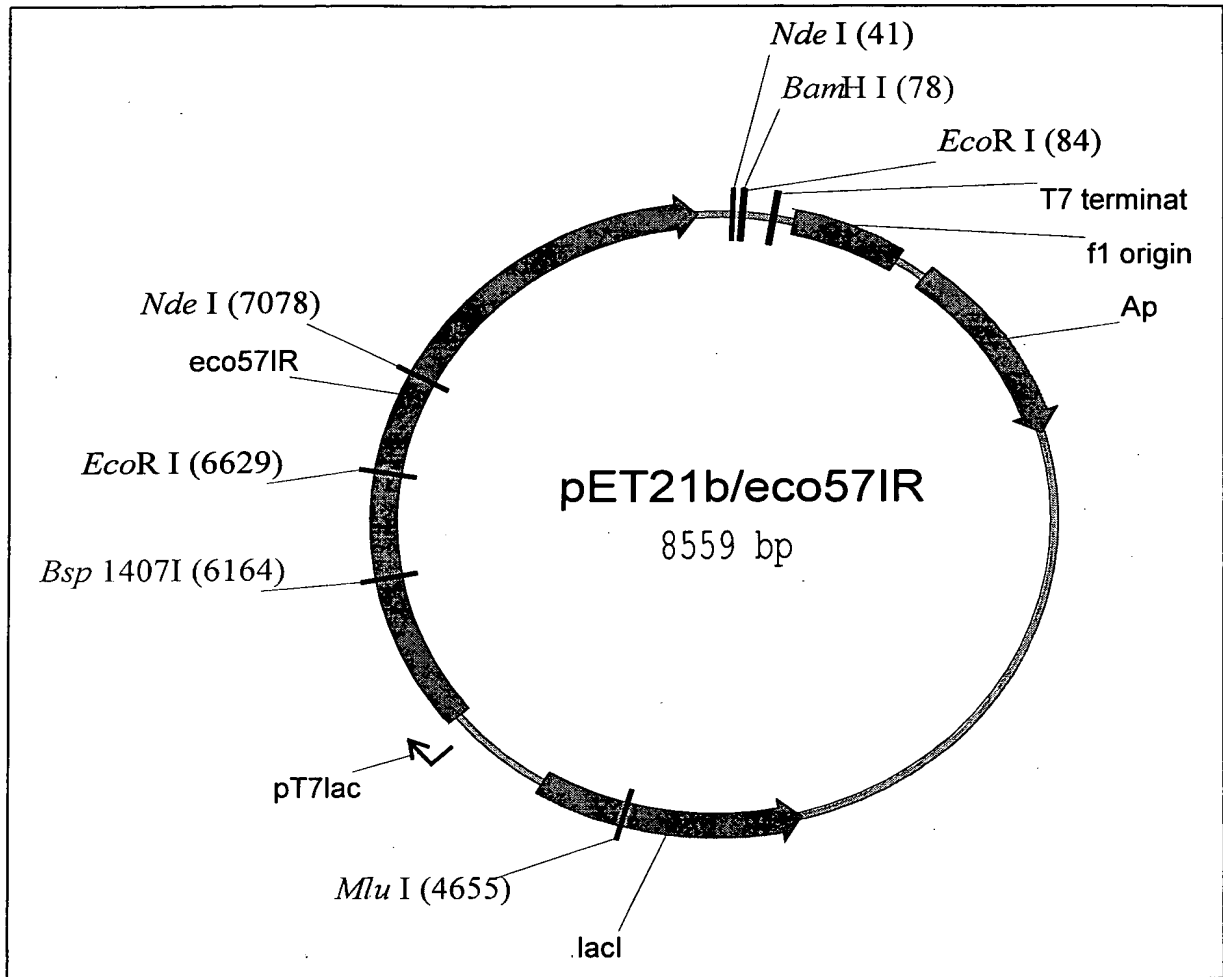Fig. 2. Schematic representation of pET21b/eco57IR D78K plasmid.

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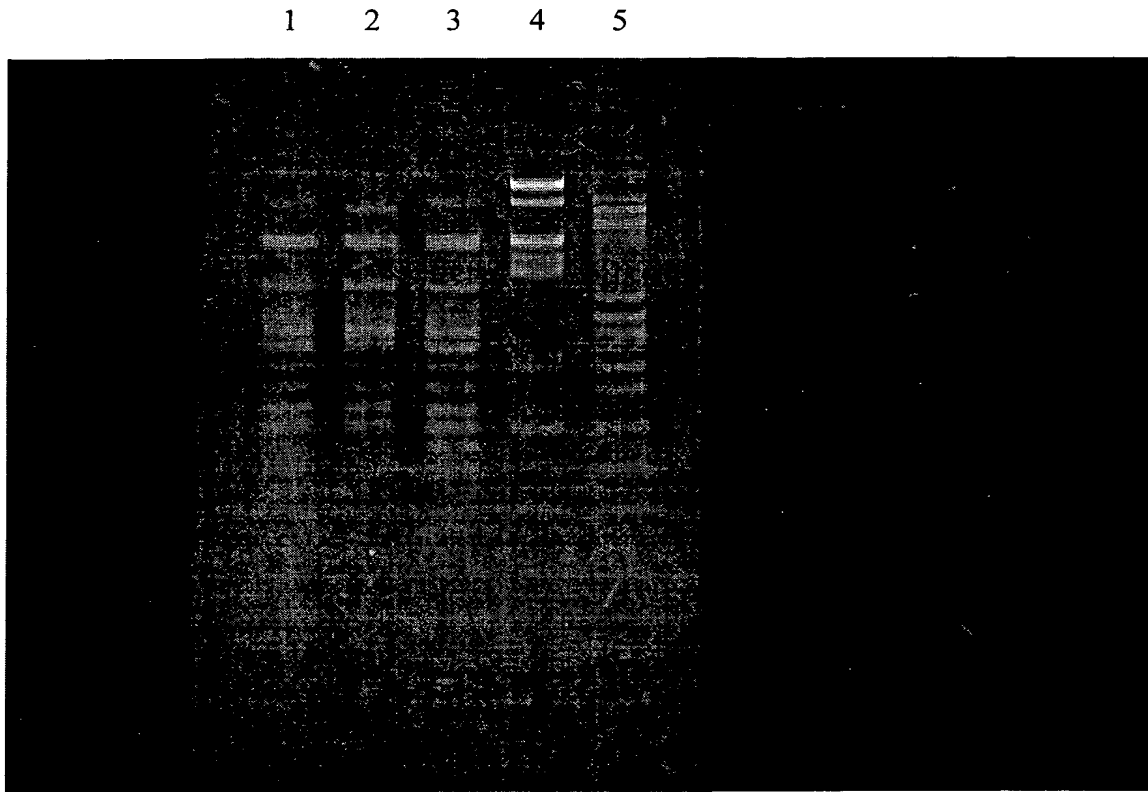Fig. 3. Schematic representation of pET21b/eco57IR plasmid.

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Fig. 4. Determination of substrate specificity of the *Eco*57I T862N mutant protein.

1. λ DNA digested with *Eco*57I+*Gsu*I+*Eco*57I T862N
2. λ DNA digested with *Eco*57I T862N
3. λ DNA digested with *Eco*57I+*Gsu*I
4. λ DNA digested with *Gsu*I
5. λ DNA digested with *Eco*57I

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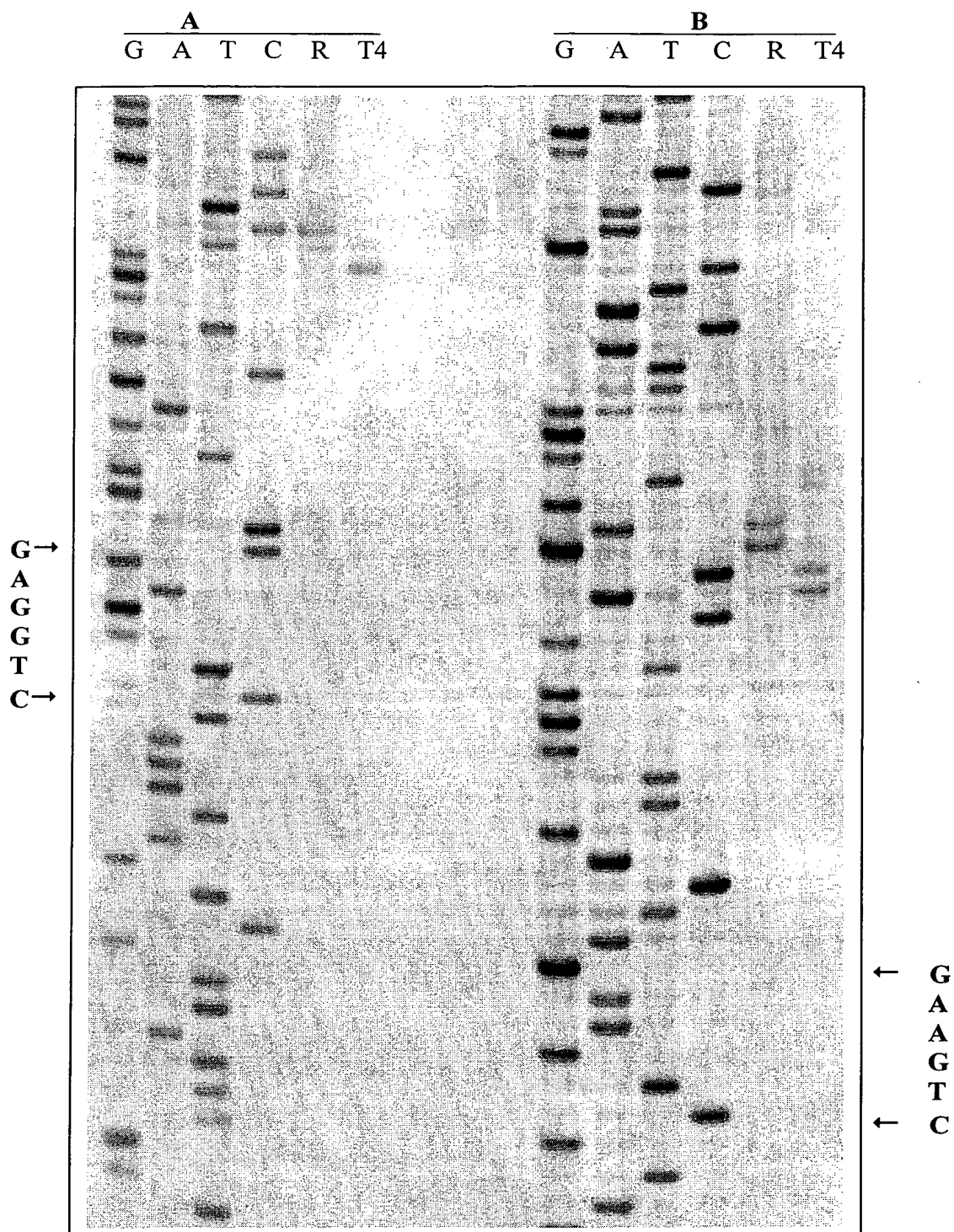
Fig. 5. A and B

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*Gsu*I site (position 454, bolded):

R.*Eco*57I T862N cleavage point (16 bases)

Direct primer (position 371)   primer extension with T7 polymerase

5'taatagactggatggaggcg...............**CTGGAG**ccggtgagcgtgggtc...........3'

3'......................................**GACCTC**ggccactcgcaccc...............5'

Cleavage point on the opposite DNA strand (14 bases)

(T4 polymerase action)
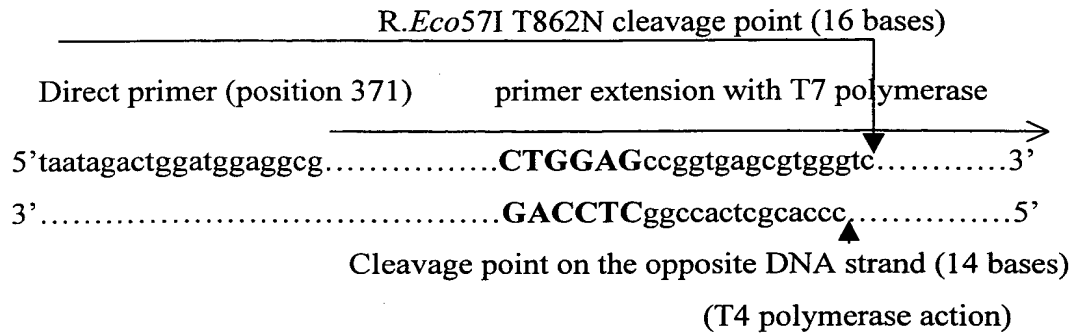
Eco57I site (position 3798, bolded):

R.*Eco*57I T862N cleavage points (16-17 bases)

Direct primer (position 3731)   primer extension with T7 polymerase

5'tcccttttttgcggcattttg................ **CTGAAG**atcagttgggtgcacg a...........3'

3'...................................... **GACTTC**tagtcaacccacgt g...............5'
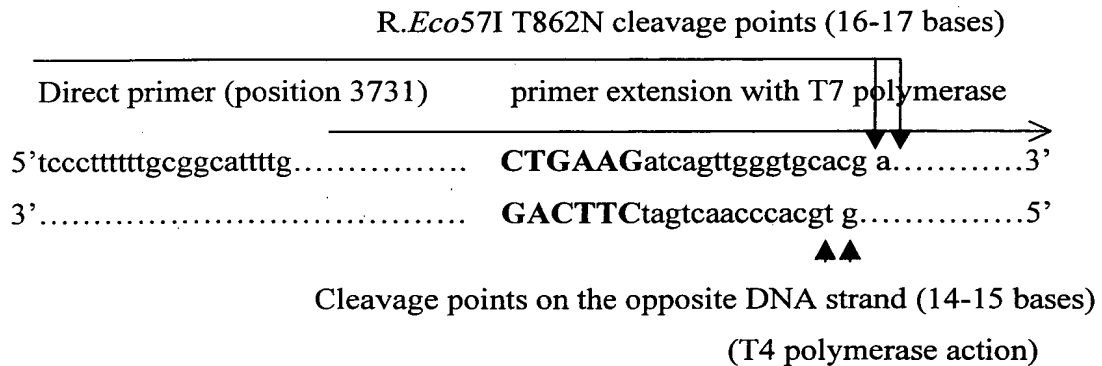
Cleavage points on the opposite DNA strand (14-15 bases)

(T4 polymerase action)

Fig. 5C

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